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ANTIBACTERIAL ACTIVITY OF PLANT EXTRACTS FROM BRAZIL AGAINST FISH PATHOGENIC BACTERIA

S.B.R. Castro[1]; C.A.G. Leal[1]; F.R. Freire[2]; D.A. Carvalho[3]; D.F. Oliveira[2]; H.C.P. Figueiredo[1]*

[1]AQUAVET – Laboratório de Doenças de Animais Aquáticos, Departamento de Medicina Veterinária, Universidade Federal de Lavras, Lavras, MG, Brasil; [2]Departamento de Química, Universidade Federal de Lavras, Lavras, MG, Brasil; [3]Departamento de Biologia, Universidade Federal de Lavras, Lavras, MG, Brasil.

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SHORT COMMUNICATION

ABSTRACT

The aim of this work was to evaluate the antibacterial activity of Brazilian plants extracts against fish pathogenic bacteria. Forty six methanolic extracts were screened to identify their antibacterial properties against *Streptococcus agalactiae*, *Flavobacterium columnare* and *Aeromonas hydrophila*. Thirty one extracts showed antibacterial activity.

Key words: plant extracts, bacteria, fish.

Fish are susceptible to several bacterial infections, mainly when reared in high densities conditions. Diseases outbreaks are responsible for elevated mortality rates and decrease of the productivity efficiency, causing high economic losses to the fish farmers (6,10). The use of antibiotics is the main treatment applied to control bacterial illness in fish farms. Due to the use of a wide variety of antibiotics, aquaculture has been implicated as potential environment to the development and selection of resistant bacteria and a source of these pathogens to other animals and humans (10,19). The adoption of same antibiotics in different fields (veterinary and human medicine) improves the emergence and occurrence of the resistance phenomenon. Some bacterial fish pathogens are also associated to diseases in humans, making the aquaculture products a potential risk to the customers (zoonotic or foodborne diseases) (24). *Streptococcus agalactiae* is a dangerous pathogen to freshwater and marine fish. The infection is characterized by brain invasion, nervous signs and septicemia (13,18). These bacteria can infect humans, causing mainly pneumonia and meningitis in newborns (17). *Aeromonas hydrophila* is responsible for cases of skin infections, septicemia and gastroenteritis in fish and human (25). *Flavobacterium columnare* is pathogenic only to freshwater fish species and shows low environmental fitness, when compared with other aquatic bacteria. Even though, this agent is highly virulent to young fish (fry and fingerling), causing skin lesions and high mortality, generally associated with poor environmental conditions (7,8). Regarding the problem of microbial resistance, there is an urgent need to establish the rules to the rational use of antibiotics and the discovery of new drugs and alternative therapies to control bacterial diseases**.** Owing the ability to synthesize many different substances, the plants are one of the top richest sources of new drugs (1,5). Extracts of Brazilian methanolic plants have a high potential as an alternative source of antibacterial compounds (15,16,20,23). Therefore, the aim of this study was to investigate the *in vitro* antibacterial activity of Brazilian plants extracts against three fish pathogenic bacteria.

During period 2002-2004, forty six native plants (Table 1) of southeast region of Brazil (San Francisco Valley and Lavras city) were sampled and identified by comparison with available specimens in the Herbarium of the Federal University of Lavras (UFLA). The fresh material were washed with distillated water, dried at 40ºC for 48h and triturated into small particles. To the extraction, 300g of particulate material was suspended in 600 ml

*Corresponding Author. Mailing address: Departamento de Medicina Veterinária, Lavras, MG, Brasil. Tel.: +55 35 3829 1714; fax: (+5535) 3829-1715. E-mail: henrique@ufla.br

Table 1. Bacterial inhibition zone (mm) of methanol extracts (0.4 mg.mL^{-1}) in agar diffusion assay.

Species	Family	Plant part assayed	Inibition zones (mm)		
			SA 16-06*[1]	FL 02-07L*[2]	AE 255-03*[3]
Actinostemon concolor (Sprengel) Müll. Arg.	Euphorbiaceae	Leaves	-	22	-
Allophylus edulis (A.St.-Hil., Cambess. & A.Juss) Radlk	Sapindaceae	Leaves	-	-	-
Amaioua guianensis Aubl.	Rubiaceae	Leaves	-	11	-
Andira fraxinifolia Benth.	Fabaceae	Leaves	-	14.5	-
Bathysa meridionalis L.B Sm. & Downs	Rubiaceae	Leaves	-	11	-
Bauhinia longifolia (Bongard) Steudel	Fabaceae	Leaves	-	11	-
Cabralea canjerana (Vell.) Mart.	Meliaceae	Stem barks	-	11	-
Calyptranthes clusiifolia (Miq.) O. Berg	Myrtaceae	Leaves	8	12.5	-
Cariniana legalis (Mart.) Kuntze	Lecythidaceae	Stem barks	-	13	13
Celtis iguanaea Jacq. Sar.	Cannabaceae	Stem barks	-	17	-
Celtis iguanaea Jacq. Sar.	Cannabaceae	Leaves	-	-	-
Croton floribundus Spreng.	Euphorbiaceae	Stem barks	-	14	11.5
Croton floribundus Spreng.	Euphorbiaceae	Leaves	7	17.5	-
Cryptocarya aschersoniana Mez	Lauraceae	Stem barks	-	15.5	-
Cupania vernalis Cambess.	Sapindaceae	Stem barks	-	12	-
Erythrina falcata Benth.	Fabaceae	Leaves	-	-	-
Eugenia florida DC.	Myrtaceae	Leaves	-	12	10
Eugenia handroana D. Legrand	Myrtaceae	Leaves	-	12	-
Guarea guidonia (L.) Sleumer	Meliaceae	Stem barks	-	17	-
Heisteria silvianii Schwacke	Olacaceae	Leaves	8	11	-
Inga marginata Willd.	Fabaceae	Stem barks	-	-	-
Machaerium hirtum (Vell.) Stellfeld	Fabaceae	Leaves	-	-	-
Matayba elaeagnoides Radlk.	Sapindaceae	Leaves	-	-	-
Matayba elaeagnoides Radlk.	Sapindaceae	Stem barks	-	-	-
Maytenus glazioviana Loes.	Celastraceae	Leaves	-	-	-
Maytenus glazioviana Loes.	Celastraceae	Stem barks	-	14	-
Merremia tomentosa (Choisy) Hallier	Convolvulaceae	Leaves	7.5	18.5	-
Mollinedia argyrogyna Perkins	Monimiaceae	Stem barks	-	-	-
Mollinedia argyrogyna Perkins	Monimiaceae	Leaves	-	10	-
Myrcia tomentosa (Aublet) DC.	Myrtaceae	Leaves	-	15.5	-
Myrcia velutina O.Berg	Myrtaceae	Stem barks	-	15	12
Pera glabrata (Schott) Poepp.	Euphorbiaceae	Leaves	-	-	-
Platycyamus regnellii Benth.	Fabaceae	Leaves	-	-	-
Protium heptaphyllum (Aubl.) Marchand	Burseraceae	Leaves	-	10	-
Protium spruceanum (Benth.) Engl.	Burseraceae	Leaves	-	13.5	-
Protium spruceanum (Benth.) Engl.	Burseraceae	Stem barks	-	10	-
Ruprechtia laxiflora Meisn.	Polygonaceae	Leaves	-	-	-
Schinus terebinthifolia Raddi	Anacardiaceae	Stem barks	-	12	-
Securinega guaraiuva Kuhlm.	Euphorbiaceae	Leaves	-	9	-
Siparuna guianensis Aubl.	Siparunaceae	Stem barks	-	11.5	-
Siparuna guianensis Aubl.	Siparunaceae	Leaves	-	-	-
Swartzia apetala Raddi.	Fabaceae	Folhas	-	-	-
Virola sebifera Aubl.	Myristiaceae	Leaves	-	14.5	-
Xylosma sp. I	Salicaceae	Leaves	-	-	-
Xylosma sp. II	Salicaceae	Leaves	-	12	-
Zanthoxylum riedelianum Engl.	Rutaceae	Leaves	7	11.5	-

(-) Inhibition not observed; *[1] *Streptococcus agalactiae*; *[2] *Flavobacterium columnare*; *[3] *Aeromonas hydrophila*.

of methanol during 48 hours. Therefore, the suspension was filtered and the extraction procedure was repeated twice to vegetable residues. The solvent was vacuum evaporated at 45ºC. The plant extracts were lyophilized and stored at -20ºC until use.

S. agalactiae (strain SA 16-06), *F. columnare* (strain BZ 01-02) and *A. hydrophila* (strain AE 255-03), isolated from *Oreochromis niloticus* (Linnaeus, 1758) were selected to the antibacterial assays (6,7,11). *Escherichia coli* ATCC 25922 was used as control (3,4).

Agar diffusion assay was performed according to the guidelines "Susceptibility testing of bacteria isolated from aquatic animals" of the Clinical and Laboratory Standards Institute (4). The strains were maintained at -70ºC. To the tests the strains were thawed and recovered by streaking onto agar plates. *A. hydrophila* and *S. agalactiae* were incubated at 30ºC for 24 hours in Müeller-Hinton (MH) Agar (Difco, USA). MH Agar was supplemented with 10% of equine blood to the cultivation of *S. agalactiae*. *F. columnare* was growth onto Medium of Hsu-Shotts (MHS) (0.3% collagen, 0.2% tryptone, 0.05% yeast extract, 0.03% calcium chloride) plates (2) for 48 hours at 26ºC. *A. hydrophila* and *S. agalactiae* suspensions were prepared in sterile 0.85% saline solution, adjusted to a turbidity of 0.5 McFarland scale, equivalent to 10^8 CFU.mL^{-1} (4). MHS broth was used to prepare the *F. columnare* suspension. The concentration of the suspension was standardized using spectrophotometer SP 11-05 (Spectrum, China) to an absorbance of 0.230, corresponding to 10^8 CFU.mL^{-1}.

The suspensions were streaked onto agar plates using sterile cotton swabs. Seven wells each with 6 mm of diameter were made in agar and 40 µL of the different extracts, diluted in ethanol: water (7:3) solutions (10 mg.mL^{-1}) were applied in the wells. Plates with *S. agalactiae* and *A. hydrophila* were incubated for 24 hours at 30ºC and plate with *F. columnare* for 48 hour at 26ºC. The inhibition zone was measured with a millimeter ruler. The assay was made in duplicate and the extracts solvent was used as control.

The extracts with antibacterial activity in agar diffusion assay were selected to determination of the minimum inhibition concentration (MIC) against the same strains. MH Broth (Difco, USA) supplemented with the divalent cations Ca^{2+} and Mg^{2+} (CAMHB) was used for strains AE 255-03 and SA 16-06 incubated at 30ºC for 24 hours. Additionally, for the cultivation of SA 16-06 CAMHB was supplemented with 2.5% of lysed horse blood (3). MHS broth was used for strain BZ 01-02 incubated at 26ºC for48 hours.

The bacterial suspensions were prepared as described in agar diffusion assay and diluted 10 times in CAHMB (3). Extract solutions were prepared in dimethyl sulfoxide (10 mg.mL^{-1}). The solutions were twofold serially diluted in CAMHB, ranging from 3000 µg.mL^{-1} to 11.71 µg.mL^{-1} and 5µL of bacterial suspension was inoculated per well.

After incubation, aqueous solution of 2, 3, 5-Triphenyltetrazolium chloride (Merck, Germany), 2 mg.mL^{-1}, was added 25 µL to each well to check the bacterial growth, indicated by the pink colour formation. Florfenicol and dimethyl sulfoxide (solvent) were used as controls.

The results of the antimicrobial screening by agar diffusion are showed in Table 1. Thirty one methanolic extracts presented antibacterial activity for at least one strain tested. *F. columnare* was the most susceptible organism, being inhibited by 31 extracts. *S. agalactiae* and *A. hydrophila* were inhibited by five and four plant extracts, respectively.

Table 2 shows the MIC values for the extracts tested. These ranged from 93.75 µg.mL^{-1} to 1500 µg.mL^{-1} for *F. columnare*. *Calyptranthes clusiifolia* (Miq.) O.Berg and *Merremia tomentosa* (Choisy) Hallier inhibited the growth of *S. agalactiae,* presenting a MIC of 1500 µg.mL^{-1}. Methanolic extracts of *Cariniana legalis* (Mart.) Kuntze, *Croton floribundus* Spreng. and *Myrcia velutina* O. Berg inhibited the growth of *A. hydrophila,* with MICs values ranged from 187.5 µg.mL^{-1} to 375 µg.mL^{-1}.

A variety of plant species are capable of synthesizing many substances with antibacterial activity. These properties have been described to extracts of many plants found in Brazilian flora (1,16,20). However, to the plants analyzed in this work, there aren't previous studies evaluating this characteristic, except to *Schinus terebinthifolia Raddi* and *Xylosma* sp. (9,12). The extract of *Schinus terebinthifolia* Raddi presented antimicrobial activity against fluorquinolone-resistant and macrolide-resistant *Staphylococcus aureus* strains. Chemical analysis showed the presence of phenols, pentacyclic, triterpenes and anthraquinonas in the extract of *Schinus terebinthifolia* Raddi (12). Species of *Xylosma* also showed the capacity of inhibit the growth of *Staphylococcus aureus* and *Candida albicans,* presenting MIC values of 2.5 mg.mL^{-1} and 1.2 mg.mL^{-1} respectively (14). Antioxidant effect, cytotoxicity and antimicrobial activity of diterpene esters and phenolic glycosides isolated from plants of the family Flacourtiaceae have been identified. However, there are no data describing the chemical composition of the other plants tested here. No one of the extracts analyzed here showed antibacterial activity against *A. hydrophila* and *S. agalactiae*, simultaneously. The occurrence of antibiotic resistant strains of bacteria has been described in aquaculture systems (6,11). Probably, the same mechanism involved in the antibiotic resistance should inhibit the deleterious action of the extracts on the bacterial cells. Even though, some extracts were effective against the pathogens, being a potential alternative to the therapy of fish diseases.

F. columnare was the microorganism most susceptible to major of tested extracts. In contrast to its high virulence to young fish, this bacterium is sensible to the main disinfectants used in fish farms, such potassium permanganate, hydrogen

Table 2. Minimal inhibitory concentration of plant methanolic extracts to selected fish bacterial pathogens.

Species	Family	Plant part	MICs values (μg.mL^{-1})		
			SA 16-06*[1]	FL 02-07L*[2]	AE 255-03*[3]
Actinostemon concolor (Sprengel) Müll. Arg.	Euphorbiaceae	Leaves	*	93.75	*
Amaioua guianensis Aubl.	Rubiaceae	Leaves	*	375	*
Andira fraxinifolia Benth.	Fabaceae	Leaves	*	375	*
Bathysa meridionalis L.B. Smith & Downs	Rubiaceae	Leaves	*	375	*
Bauhinia longifólia (Bongard) Steudel	Fabaceae	Leaves	*	750	*
Cabralea canjerana (Vell.) Mart.	Meliaceae	Stem barks	*	187.5	*
Calyptranthes clusifolia (Miq.) O. Berg	Myrtaceae	Leaves	1500	187.5	*
Cariniana legalis (Mart.) Kuntze	Lecythidaceae	Stem barks	*	93.75	187.5
Celtis iguanaea Jacq. Sarg.	Cannabaceae	Stem barks	*	187.5	*
Croton floribundus Spreng.	Euphorbiaceae	Stem barks	*	93.75	375
Croton floribundus Spreng.	Euphorbiaceae	Leaves	-	93.75	*
Cryptocarya aschersoniana Mez	Lauraceae	Stem barks	*	93.75	*
Cupania vernalis Cambess.	Sapindaceae	Stem barks	*	750	*
Eugenia florida DC.	Myrtaceae	Leaves	*	375	1500
Eugenia handroana D. Legrand	Myrtaceae	Leaves	*	187.5	*
Guarea guidonia (L.) Sleumer	Meliaceae	Stem barks	*	187.5	*
Heisteria silvianii Schwacke	Olacaceae	Leaves	-	750	*
Maytenus glazioviana Loes.	Celastraceae	Stem barks	*	187.5	*
Merremia tomentosa (Choisy) Hallier	Convolvulaceae	Leaves	1500	93.75	*
Mollinedia argyrogyna Perkins	Monimiaceae	Leaves	*	375	*
Myrcia tomentosa (Aublet) DC.	Myrtaceae	Leaves	*	93.75	*
Myrcia velutina O.Berg	Myrtaceae	Stem barks	*	187.5	375
Protium heptaphyllum (Aubl.) Marchand	Burseraceae	Leaves	*	750	*
Protium spruceanum (Benth.) Engl.	Burseraceae	Leaves	*	375	*
Protium spruceanum (Benth.) Engl.	Burseraceae	Stem barks	*	375	*
Schinus terebinthifolia Raddi	Anacardiaceae	Stem barks	*	187.5	*
Securinega guaraiuva Kuhlm.	Euphorbiaceae	Leaves	*	1500	*
Siparuna guianensis Aubl.	Siparunaceae	Stem barks	*	93.75	*
Virola sebifera Aubl.	Myristiaceae	Leaves	*	93.75	*
Xylosma sp. II	Salicaceae	Leaves	*	375	*
Zanthoxylum riedelianum Engler	Rutaceae	Leaves	-	1500	*
Florfenicol		-	2	1	2

(*) MIC assay not done; (-) No MIC values established; *[1] *Streptococcus agalactiae*; *[2] *Flavobacterium columnare*; *[3] *Aeromonas hydrophila*.

peroxide, chloramines and salt (21,22). Despite of their common use, these compounds may be dangerous to fry and aquatic environment. The plant extracts can be applied as an alternative to prevent and control outbreaks of columnaris, mainly in hatchery. Since these substances are natural, their hazardous potential is lower when compared with other products. The results show that analyzed plants presented a high potential as alternative therapy of bacterial fish diseases currently observed in Brazilian fish farming.

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RESUMO

Atividade antibacteriana de extratos de plantas do Brasil contra bactérias patogênicas para peixes

O objetivo deste trabalho foi avaliar a atividade antibacteriana de extratos de plantas brasileiras contra bactérias patogênicas para peixes. A atividade antibacteriana de quarenta e seis extratos metanólicos de plantas foi avaliada contra os agentes *Streptococcus agalactiae*, *Flavobacterium columnare* e *Aeromonas hydrophila*. Trinta e um extratos apresentaram atividade antibacteriana.

Palavras-chave: extratos de plantas, bactérias, peixes.

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